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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/2 (2)

Diagnostics
Diagnostic

PM 7/2 (2) *Tobacco ringspot virus*

Specific scope

This Standard describes a diagnostic protocol for *Tobacco ringspot virus*[1]

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols.*

Specific approval and amendment

First approved in 2000-09.
Revision approved in 2017-03

1. Introduction

Tobacco ringspot virus (TRSV) is the type member of the genus *Nepovirus* within the family Secoviridae. It has isometric particles of approximately 28 nm and is readily transmitted by sap inoculation. It is transmitted in nature by the nematode vector *Xiphinema americanum* and closely related *Xiphinema* species. *Thrips tabaci*, spider mites, grasshoppers and aphids have also been reported as possible vectors (Stace-Smith, 1985) but their significance for the natural spread of TRSV is currently unclear. The same holds true for *Apis mellifera* (the European honeybee), in which TRSV was found to systemically spread and propagate (Li *et al.*, 2014). TRSV is transmitted by seed in several host plants. It naturally infects a wide range of herbaceous and woody hosts, causing many serious diseases in the regions of North America where the nematode vectors occur. The most significant crops affected by TRSV are soybean (*Glycine max*), grapevine (*Vitis vinifera*) and blueberry (*Vaccinium corymbosum*) and, to a lesser extent, tobacco (*Nicotiana tabacum*), a number of ornamentals and Cucurbitaceae. Its host range is similar to that of *Tomato ringspot virus* (ToRSV), but it is less important on fruit crops generally; it does, however, pose a significant risk to *Vaccinium* and *Vitis* within the EPPO region. TRSV has been reported from many parts of the world. In the EPPO region it has been found in ornamentals, but most of these reports were associated with movement of material originating from North America. In particular, it has been recorded in several EPPO countries but these reports are either unconfirmed or concern detection in imported material.

[1]Use of brand names of chemicals or equipment in this EPPO Standard implies no approval of them to the exclusion of others that may also be suitable.

A flow diagram describing the procedure for detection and identification of TRSV is given in Fig. 1.

2. Identity

Name: *Tobacco ringspot virus*
Synonym: Anemone necrosis virus, blueberry necrotic ringspot virus
Taxonomic position: Viruses: *Picornovirales*: *Secoviridae*: *Comovirinae*: *Secoviridae*: *Nepovirus*
EPPO Code: TRSV00
Phytosanitary categorization: EPPO A2 List no. 228; EU Annex I/A1

3. Detection

3.1 Symptoms

In addition to the crops already mentioned, TRSV has been found in *Anemone*, *Bacopa*, *Begonia semperflorens*, *Capsicum annuum*, *Carica papaya*, *Citrillus lanatus*, *Cornus*, *Cucumis maxima*, *Cucumis melo*, *Cucumis pepo*, *Cucumis sativus*, *Daphne*, *Fraxinus*, *Gladiolus*, *Hemerocallis*, *Hydrangea*, *Impatiens walleriana*, *Iris*, *Lobelia*, *Lupinus*, *Malus domestica*, *Mentha*, *Narcissus*, *Pelargonium*, *Petunia*, *Portulaca*, *Phlox subulata*, *Prunus avium*, *Prunus incisa*, *Prunus persicae*, *Prunus serrula*, *Prunus serrulata*, *Rubus fruticosus*, *Rubus* sp., *Sambucus*, *Solanum lycopersicum*, *Solanum melongena*, *Vaccinium corymbosum*, *Vigna unguiculata* and various weeds (Németh, 1986; Stace-Smith, 1987; EPPO/CABI, 1997; NVWA, 2006, 2010; Coneva *et al.*, 2008; Fuchs *et al.*, 2010; Abdalla *et al.*, 2012; Sneideris *et al.*, 2012; Kundu *et al.*, 2015).

In soybean, terminal buds are curved (bud blight), with other buds progressively becoming brown and brittle.

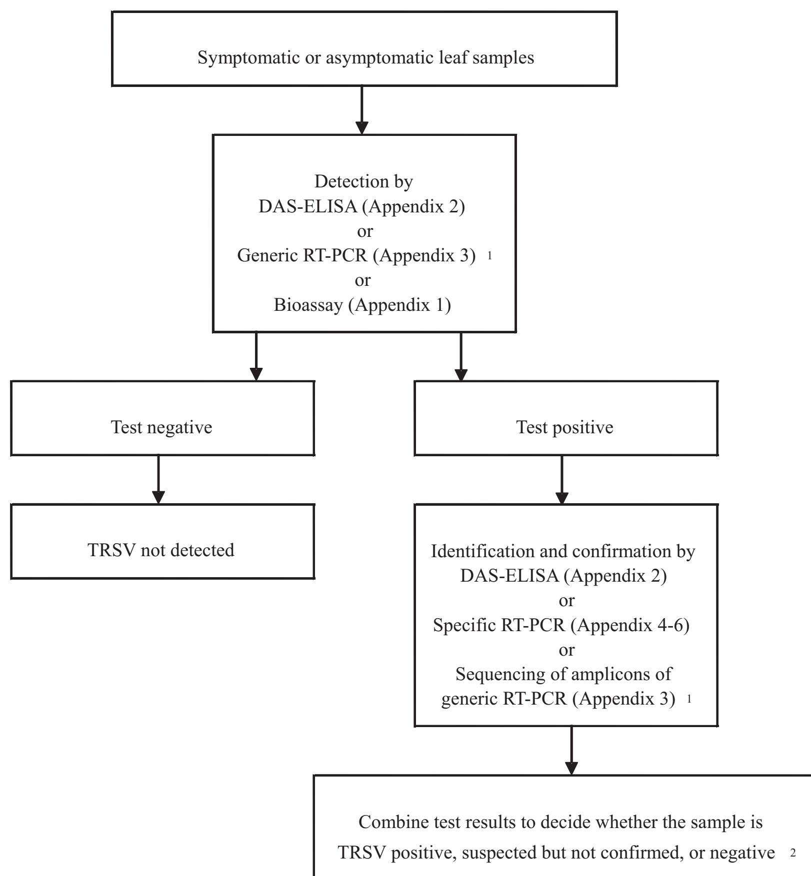

Fig. 1 Possible combinations of tests for the detection and identification of TRSV. Since none of the molecular tests has been completely validated, the interpretation of test results is critical and additional tests are needed for confirmation.
[1]Note that the generic RT-PCR has been validated for detection. Therefore, sequence analysis of the amplicon might not be sufficient for identification and confirmation by another test might be required in specific situations.
[2]This statement is made because of the lack of validation data for the molecular tests and risks of cross-reactions.

Brown streaks may develop on the stems and petioles of larger leaves and pods are underdeveloped and aborted. Pods that set before infection may develop dark blotches.

On grapevine, the new growth is weak and sparse, internodes are shortened, leaves are small and distorted, and plants are stunted with very few berries produced and these berries are deformed. It should be noted, that grapevines are often infected by several viruses at the same time, which might influence the symptomatology.

On blueberry, the symptoms include stunting, twig dieback, necrotic or chlorotic spots, and rings or line patterns on the foliage.

On tobacco, TRSV causes ring and line patterns on the foliage, and stunting.

In cucurbits, stunting with leaf mottle occurs and the fruit is deformed.

On tomatoes, leaves of infected plants may display chlorosis and curling of the margins (Ontario CropIPM, 2009). In addition, discoloration and rugosity may be observed on the surface of and inside infected fruits.

TRSV has only been seen a few times in cherry, in which scattered young leaves throughout the crown show irregular blotching over the whole leaf blade and have deformed margins.

In wild *R. fruticosus*, there are faint to severe ringspots, mottling and mosaic, yellow line patterns, leaf distortion and stunting of infected foliage (Stace-Smith, 1987). TRSV has only been found once in a cultivated *Rubus* species.

No definite symptoms have been associated with TRSV in pelargonium.

3.2 Sampling

TRSV generally occurs in all plant parts (including roots and seeds) of its host species. Detection is, however, easiest from soft tissues (e.g. young leaves and shoots). For this reason, it is preferable to collect samples during an appropriate period in the growing season of the host species concerned to ensure an adequate concentration of virus. For pelargonium, for example, tests are best done between November and April, or when temperatures are cooler. There is no experience in the region with testing of seeds or pollen and for testing seeds these are germinated first.

3.3 Screening tests

For detection of TRSV the following tests are recommended.

3.3.1 DAS-ELISA

Double antibody sandwich (DAS)-ELISA is the preferred method of detection and identification. Instructions to perform an ELISA test are provided in EPPO Standard PM 7/

125 *ELISA tests for viruses* (EPPO, 2015a) and further information is provided in Appendix 2.

3.3.2 Molecular tests

For detection, the RT-PCR tests with generic primers for *Nepovirus* subgroup A (Wei & Clover, 2008; Appendix 3) and TRSV-specific primers as described under identification can be used. Digiaro *et al.* (2007) described primers for conventional RT-PCR for generic detection of nepoviruses belonging to different subgroups and specific detection of TRSV. However, since these tests are not widely used and experience with them is limited in the EPPO region, they are not described in this protocol.

3.3.3 Bioassay

Mechanical inoculation to herbaceous test plants is a simple method for virus detection. It does not lead to specific identification of TRSV when used alone, as the symptoms produced on test plants are generally the same for all nepoviruses. However, test-plant inoculations can be used for virus detection and subsequent identification by other methods. The bioassay is described in Appendix 1.

3.3.4 Other tests

Electron microscopy (EM), in combination with symptoms, can give an indication of the presence of TRSV, when isometric particles with a diameter of approximately 28 nm are observed. Instructions to perform EM are provided in EPPO Standard PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015b).

4. Identification

DAS-ELISA and molecular tests are recommended for identification of TRSV.

4.1 DAS-ELISA

For TRSV several commercial antisera are available, and therefore ELISA can be used to detect and identify the virus in herbaceous hosts, grapevine and blueberry using appropriate extraction buffers. In general, it is recommended to follow the protocol provided by the supplier of the antiserum. Note that the source of antibodies is critical because cross-reactions with other nepoviruses can occur. Further details on DAS-ELISA can be found in EPPO Standard PM 7/125 (EPPO, 2015a) and Appendix 2.

4.2 Molecular methods

4.2.1 PCR tests

Several conventional and real-time RT-PCR tests have been described for identification of TRSV (Jossey & Babadoost, 2006; Yang *et al.*, 2007; Martin *et al.*, 2009; Fuchs *et al.*, 2010). As currently complete validation data are not available for any of these tests, the following tests were selected on the basis of their relative performance under described conditions:

- Conventional RT-PCR test from Jossey & Babadoost (2006) described in Appendix 4
- Real-time RT-PCR test from Yang *et al.* (2007), described in Appendix 5.

Furthermore, a conventional RT-PCR test developed and successfully used by M. Zeidan (PPIS, NL) is described in Appendix 6.

4.2.2 Sequencing

TRSV can be identified by sequence analysis of the amplicon obtained by the generic RT-PCR from Wei & Clover (2008), as described in Appendix 3, following the guidelines described in Appendices 7 and 8 of the EPPO Standard 7/129, *DNA barcoding as an identification tool for a number of regulated pests* and according to the species demarcation criteria of the International Committee on Taxonomy of Viruses (Sanfaçon *et al.*, 2012). Note that in specific situations an additional test might be needed for confirmation of the identity.

4.3 Other tests

Immunoelectron microscopy (IEM) is a quick technique and is described in EPPO Standard PM 7/126 (EPPO, 2015b). Note that the source of antibodies is critical because cross-reactions between nepoviruses can occur. Further details are described in EPPO Standard PM 7/126 *Electron microscopy in diagnosis of plant viruses* (EPPO, 2015b). Antisera are described in Appendix 2.

5. Reference material

Reference isolates of TRSV are available from:

American type culture collection (ATCC): http://www.lgcstandards-atcc.org/en/Support/LGC_Contact_Us.aspx

Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstraße 7B, 38124, Braunschweig, Germany (https://www.dsmz.de/catalogues/catalogue-plant-viruses-and-antisera.html)

National Plant Protection Organization of the Netherlands (NPPO-NL), PO Box 9102, 6700 HC Wageningen, the Netherlands.

Specifications and characteristics of available isolates can be found via Q-bank: http://www.q-bank.eu

Wageningen University and Research (WUR), Postbus 16, 6700 AA Wageningen, the Netherlands.

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on the different tests for this organism can be obtained from:

Fera, Plant Health Diagnostics and Advice, Sand Hutton, York YO41 1LZ, United Kingdom (for DAS-ELISA)

French Agency for Food, Environmental and Occupational Health and Safety (ANSES), Plant Health Laboratory, Virology Team, 7 rue Jean Dixméras, 49044 Angers, Cedex 01, France (for Bioassay and PCR detection)

Julius Kühn-Institut, Messeweg 11-12, 38104 Braunschweig, Germany (for IEM and DAS-ELISA)

Naktuinbouw Laboratories, Sotaweg 25, PO Box 135, 2370 AC Roelofarendsveen, the Netherlands (for Bioassay and DAS-ELISA)

National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands (for Bioassay and DAS-ELISA).

9. Feedback on this diagnostic protocol

If you have any feedback concerning this diagnostic protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

11. Acknowledgements

This diagnostic protocol was originally drafted by: D. M. Wright, Central Science Laboratory, Sand Hutton, York YO41 1LZ (GB) and H. J. Vetten, Institut für Pflanzenvirologie, Mikrobiologie und Biologische Sicherheit, Biologische Bundesanstalt für Land- und Forstwirtschaft, Messeweg 11-12, 38104 Braunschweig (DE). It was revised by J. W. Roenhorst, National Plant Protection Organisation, PO Box 9102, 6700 HC Wageningen (NL).

This protocol was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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Appendix 1 – Biossay

Different types of plant material can be used for mechanical inoculation of test plants. Note that for some crops the success of inoculation may depend on the composition of the extraction buffer.

Extraction buffers to be used for test-plant inoculations

The following buffers can be used to prepare inoculum from various plants for mechanical inoculation of test plants:

- For most plant species, the infected material may be ground in 0.02 M Na/K phosphate buffer, pH 7.0, containing 2% (w/v) polyvinylpyrrolidone (PVP) (MW 10 000 to 40 000);
- For extraction from grapevine or blueberry, an aqueous solution of nicotine may be used at the following concentrations: for grapevine 2.5% nicotine and for blueberry 2.0% nicotine or, alternatively, 0.5 M Tris-HCl buffer, pH 8.2, containing 2% PVP MW 10 000 to 40 000;
- For *Pelargonium* grinding in 0.06 M phosphate buffer, pH 7.6, containing 4% polyethylene glycol (PEG) (MW 6000) is recommended.

Celite is added to the inoculum as an abrasive. Alternatively, carborundum can be used to dust leaves prior to inoculation. The abrasive should be washed off after inoculation to avoid damage to the inoculated leaves, which will mask symptoms. Prior to inoculation, plants may be maintained for 12 h in the dark. Inoculated plants should be kept at 18–22°C in a glasshouse or growth chamber. Further details on quality control for mechanical inoculation of test plants are described by Roenhorst *et al.* (2013).

Recommended test plants and their symptoms[2]

Chenopodium amaranticolor and *Chenopodium quinoa*: necrotic local lesions; systemically chlorotic and/or necrotic spots and/or dieback; systemic infections are not always induced.

Nicotiana benthamiana, *Nicotiana clevelandii*, *Nicotiana occidentalis*-P1 and *Nicotiana tabacum*: necrotic local lesions may develop into rings or ringspots; systemically infected leaves usually show ring or line patterns.

Phaseolus vulgaris: necrotic spots on inoculated leaves; systemically infected leaves may show spots and rings. The growing tip may become necrotic.

Cucumis sativus: chlorotic or necrotic local lesions; systemic mottling and dwarfing with apical distortion.

[2]Note that symptoms may vary with respect to virus isolate, test-plant accession and climatic conditions.

Appendix 2 – Data on antisera for DAS-ELISA and IEM

Instructions to perform an ELISA test and IEM are provided in EPPO Standards PM 7/125 *ELISA tests for viruses* and PM 7/126 *Electron microscopy in diagnosis of plant viruses*, respectively.

The source of antibodies is critical. Several TRSV antisera are available from various suppliers and may differ in reactivity. In general it is recommended to follow the protocol provided by the supplier of the antiserum. In a comparative test, commercial antisera from Agdia, DSMZ and Prime Diagnostics reacted with 15 different TRSV isolates from different host plants and origins (E. Meekes, 2008, unpublished data).

The antiserum of Prime Diagnostics has been recently validated for testing leaf material of *N. occidentalis*-P1 by the NPPO in the Netherlands. Following the instructions of the supplier, the test could detect all four included TRSV isolates from different host plants and origins up to a relative infection rate of 0.1%. Cross-reaction was observed with the closely related *Potato black ringspot virus*. No cross-reaction occurred with other nepoviruses, i.e. *Arabis mosaic virus*, *Arracacha virus B* (tentative member), *Beet ringspot virus*, *Cherry leaf roll virus*, *Raspberry ringspot virus*, *Tomato black ring virus*, *Tomato ringspot virus* and the as-yet unassigned *Strawberry latent ringspot virus*. No background reaction was obtained for leaf material of *N. occidentalis*-P1.

In 2012, Naktuinbouw also validated the Prime Diagnostic antiserum for use in different ornamental crops. TRSV-infected leaf material of *N. benthamiana* was spiked at a ratio of 1:10 to healthy leaf material of different plant species, i.e. *Celosia* sp., *Hemerocallis fulva*, *Hosta* sp., *Iris* sp., *Lobelia* sp., *N. benthamiana*, *Pentas* sp., *Petunia hybrida* and *Phlox paniculata*. In these spiked samples, TRSV could be detected up to a relative infection rate of approximately 0.1%. No matrix effects were observed for any of these plant species. For *Pelargonium* sp. and *Verbena* sp., however, matrix effects (background) could occur. With regard to the specificity, no cross-reactions occurred with the following viruses *Angelonia flower break virus*, *Alfalfa mosaic virus*, *Arabis mosaic virus*, *Broad bean wilt virus*, *Calibrachoa mottle virus*, *Cucumber mosaic virus*, *Chrysanthemum virus B*, *Hosta virus X*, *Impatiens necrotic*

spot virus, *Lettuce mosaic virus*, *Pelargonium flower break virus*, *Pelargonium line pattern virus*, *Potato virus Y*, *Scrophularia mottle virus*, *Nemesia ring necrosis virus*, *Tomato aspermy virus*, *Tomato black ring virus*, *Tomato bushy stunt virus*, *Tobacco mosaic virus*, *Tobacco necrosis virus*, *Tomato mosaic virus*, *Tomato ringspot virus*, *Tobacco rattle virus* and *Tomato spotted wilt virus*. In addition, in earlier studies no cross-reaction was observed for *Grapevine fanleaf virus* (E. Meekes, 2008, unpublished data). Repeatability and reproducibility were 100%.

Appendix 3 – One step RT PCR (adapted from Wei & Clover, 2008)

1. General information

1.1 Two tests are used for generic detection of nepoviruses of subgroups A and B. TRSV belongs to subgroup A and therefore only this test will be described. The original two-step RT-PCR described by Wei & Clover (2008) is adapted to a one-step format for which the choice of the reagents appeared critical (P. Gentit, pers. comm.).

1.2 The test can be performed on fresh or freeze-dried leaves of various crop and test plant species (see Wei & Clover, 2008, Table 1)

1.3 For subgroup A, the target sequences are two conserved motifs 'TSEGYP' and 'LPCQVGI' in the RNA-dependent RNA polymerase (RdRp) gene, producing an amplicon of approximately 340 bp.

1.4 Oligonucleotides

Primer	Sequence*
NepoA-F–Flap	5′-AATAAATCATAAACDTCWGARGGITAYCC-3′
NepoA-R	5′-RATDCCYACYTGRCWIGGCA-3′

*Degenerate nucleotide sequence according to IUPAC nucleic acid notation. The underlined section corresponds to the Flap extension (Wei & Clover, 2008).

1.5 The test has been successfully performed on a PCR thermocycler Labcycler (Sensoquest).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 100 mg of plant material is used for total RNA extraction using the RNeasy® Plant Mini Kit from Qiagen. Alternative procedures may work as well.

2.1.2 Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or –80°C for longer periods.

2.2. One-step reverse transcription PCR

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	6.1	N.A.
RT-PCR buffer (Invitrogen)	2×	12.5	1×
$MgSO_4$	50 mM	0.4	0.8 mM
Forward primer: NepoA-F–Flap	10 µM	1.75	0.7 µM
Reverse primer: NepoA-R	10 µM	1.75	0.7 µM
RT/Taq Platinum® polymerase (Invitrogen)		0.5	
Subtotal		23.0	
RNA extract		2.0	
Total		25.0	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 One step RT-PCR using Super Script® III Platinum One Step qRT-PCR System (Invitrogen).

2.2.3 RT-PCR cycling parameters: reverse transcription step at 50°C for 30 min; denaturation at 94°C for 3 min; 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, elongation at 69°C for 30 s, elongation at 72°C for 5 min.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix, or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue, host tissue spiked with the target organism, or lyophilized infected plant material).
- Negative amplification control (NAC): molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue or a synthetic control

(e.g. cloned PCR product[3]). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons
- PIC, PAC should produce amplicons of approximately 340 bp

When these conditions are met:

- A test will be considered positive if amplicons of approximately 340 bp are produced
- A test will be considered negative if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained

4 Performance criteria available

The generic one-step RT-PCR for detection of nepoviruses of subgroup A has been validated at ANSES (FR). The performance characteristics are described below. Further details can be found in the EPPO Database on Diagnostic Expertise, i.e. validation data for diagnostic tests.

4.1 Analytical sensitivity data

Subgroup A primers were able to detect 10^3–10^5 dilutions of RNA extracted from different virus isolates in RNA from healthy plant material.

4.2 Analytical specificity data

Subgroup A primers detected all five tested isolates belonging to subgroup A: *Arabis mosaic virus* (ArMV), *Grapevine fan leaf virus* (GFLV), *Potato black ringspot virus* (PBRSV), *Tobacco ringspot virus* (TRSV) and *Raspberry ringspot virus* (RpRSV).

No cross-reactions were observed with two isolates belonging to subgroup B, *Beet ringspot virus*, *Tobacco black ring virus* (TBRV), and five isolates of species belonging to subgroup C, *Apricot latent ringspot virus*, *Cherry leaf roll virus* (CLRV), *Myrobolan latent ringspot virus*, *Peach rosette mosaic virus* (PRMV) and *Tomato ringspot virus* (ToRSV). Cross-reaction was observed with an isolate of *Artichoke yellow ringspot virus*, belonging to subgroup C.

Furthermore, no background was observed in healthy plants of six different species, *Cucumis sativus* (cucumber), *Prunus avium* (cherry), *Prunus persicae* (peach), *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Vitis vinifera* (grapevine), and no cross-reactions with *Plum pox virus* and *Strawberry latent ringspot virus* on peach, and *Pepino mosaic virus*, *Tomato infectious chlorosis virus* and *Tomato chlorosis virus* (PepMV) on tomato.

[3]Laboratories should take additional care to prevent risks of cross-contamination when using cloned PCR products.

In addition, the following data on the analytical specificity are available from the original two-step RT-PCR format as described by Wei & Clover (2008).

Subgroup A primers detected all ten tested isolates belonging to subgroup A: ArMV (one isolate), GFLV (four), PBRSV (one), TRSV (three), RpRSV (one).

No cross-reactions were observed with seven isolates belonging to subgroup B: *Cocoa necrosis virus* (one isolate), *Cycas necrotic stunt virus* (three) and TBRV (three), and four isolates of species belonging to subgroup C: *Blackcurrant reversion virus*, CLRV, PRMV and ToRSV.

Furthermore, no background reaction was observed in healthy plants of 15 different species: *Dianthus caryophyllus* (carnation), *Castanea sativa* (chestnut), *Humulus lupulus* (hop), *Juglans regia* (walnut), *Ipomoea batatas* (sweet potato), *Malus* × *domestica* (apple), *Solanum muricatum* (pepino), *Solanum tuberosum* (potato), *Prunus avium* (cherry), *Ribes nigrum* (blackcurrant), *Fragaria vesca* (strawberry), *Solanum lycopersicum* (tomato), *Tropaeolum majus* (nasturtium), *Vitis riparia* (grapevine), *Vitis vinifera* (grapevine).

4.3 Data on repeatability

Each test was repeated 3 times. The level of repeatability reached for the RT-PCR targeting subgroup A of the genus *Nepovirus* was 94.1%.

4.4 Data on reproducibility

Not available.

Appendix 4 – Two-step Conventional RT PCR (Jossey & Babadoost, 2006)

1. General information

1.1 This test is used for specific detection and identification of TRSV, using primers originally designed by Jossey & Babadoost (2006).

1.2 This test has been successfully used for testing *Allium sativum*, *Begonia*, *Capsicum annuum*, *Chrysanthemum*, *Cucumis sativus*, *Cucurbita pepo*, *Cyclamen*, *Fragaria* × *ananassa*, *Fuchsia*, *Gerbera*, *Gloxinia*, *Hyacinthus*, *Hemerocallis*, *Impatiens*, *Iris*, *Ixia polystachya*, *Lilium*, *Narcissus*, *Pelargonium*, *Petunia*, *Primula*, *Ruellia*, *Solanum lycopersicum*, *Solanum melongena*, *Solanum tuberosum*, *Streptocarpus*, *Tagetes*, *Tulipa*, *Viola odorata.*

1.3 The primers anneal to the conserved region in the coat protein gene, producing an amplicon of approximately 348 bp. The host sequence and exact location of the amplicon are not known.

1.4 Oligonucleotides

Primer	Sequence
TRSV-F	5′-CTTGCGGCCCAAATCTATAA-3′
TRSV-R	5′-ACTTGTGCCCAGGAGAGCTA-3′

1.5 The test has been successfully performed on a Bio-Rad C1000 Touch™ Thermal Cycler and an Eppendorf Mastercycler Personal Thermal Cycler.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 One gram of symptomatic plant material is used for total RNA extraction using the RNeasy Mini Kit (Qiagen).

2.1.2 Extracted RNA should be stored refrigerated for short-term storage (<8 h) at –20°C (<1 month) or at –80°C for longer periods.

2.2 Reverse transcription (RT; to produce cDNA from RNA)

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	N.A.	3.0	N.A.
RT buffer (Evrogen)	5×	4.0	1×
dNTPs (each)	10 mM	2.0	1.0 mM
Dithiothreitol (DTT)	20 mM	2.0	2.0 mM
Random decamer primers	20 μM	3.0	3.0 μM
Reverse transcriptase (Evrogen; MMLV)	100 U/μL	1.0	5 U
Subtotal		15.0	
RNA		5.0	
Total		20.0	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free.

2.2.2 RT step using MMLV reverse transcriptase (Evrogen, Russia).

2.2.3 RT conditions: incubation at 40°C for 40 min followed by a denaturation step at 70°C for 10 min.

2.3 Conventional PCR

2.3.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	N.A.	11.0	N.A.
Mas TaqMix CF (Dialat Ltd)	5×	5.0	1×
Forward primer: TRSV-F	10 μM	2.0	0.8 μM
Reverse primer: TRSV-R	10 μM	2.0	0.8 μM
Subtotal		20.0	
cDNA derived from the RT-step		5.0	
Total		25.0	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free.

2.3.2 PCR using a hot-start Taq DNA polymerase (Dialat Ltd, Russia).

2.3.3 PCR cycling parameters: denaturation at 96°C for 5 min; 35 cycles consisting of denaturation at 96°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s; terminal elongation at 72°C for 5 min.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue, host tissue spiked with the target organism, or lyophilized infected plant material).
- Negative amplification control (NAC): molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[4]). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons

[4]Laboratories should take additional care to prevent risks of cross-contamination when using cloned PCR products.

- PIC, PAC should produce amplicons of approximately 348 bp

When these conditions are met:

- A test will be considered positive if amplicons of approximately 348 bp are produced
- A test will be considered negative, if it produces no band or a band of a different size
- Tests should be repeated if any contradictory or unclear results are obtained

4 Performance criteria available

No validation data is available.

Appendix 5 – One-step real-time RT-PCR (adapted from Yang *et al.*, 2007)

1. General information

1.1 This test is used for specific detection and identification of TRSV, using primers and probe described by Yang *et al.* (2007). The protocol has been successfully applied and slightly adapted by IVIA, Valencia (ES).

1.2 This test has been successfully used for testing *Prunus avium*, *Rubus fruticosus*, *Vaccinium* spp. and *Vitis vinifera*.

1.3 The primers and probe target the coat protein gene, producing an amplicon of approximately 91 bp. Primers and probe sequences based on CP sequences of NCBI GenBank accession numbers AF461163.1, AF461164.1, L09205.1, AY363727.1, covering nucleotides 3202–3220, 3236–3252, 3273–3292, in TRSV RNA 2 complete sequence, NCBI Sequence AY363727.1.

1.4 Oligonucleotides

Primer/Probe	Sequence
TRSV-FP	5′-GGGGTGCTTACTGGCAAGG-3′
TRSV-RP	5′-GCACCAGCGTAAGAACCCAA-3′
TaqMan-MGB TRSV FAM Probe	5′-FAM-TGATTTGCGGCGTACTG-MGB-3′

1.5 The test has been successfully performed on a StepOnePlus real-time PCR system (Applied Biosystems).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Up to 50 mg leaf material is used for RNA extraction by using the PowerPlant RNA isolation kit (MOBIO), or alternative RNA extraction procedures.

2.1.2 Extracted RNA should be stored refrigerated for short-term storage (<8 h) at –20°C (<1 month) or at –80°C for longer periods.

2.2 One-step real-time reverse transcription polymerase chain reaction

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	N.A.	1.08	N.A.
Real-time RT PCR master mix	2×	6.0	1×
PCR forward primer: TRSV-FP	10 μM	0.6	0.5 μM
PCR reverse primer: TRSV-RP	10 μM	0.6	0.5 μM
Probe: TaqMan-MGB TRSV FAM	5 μM	0.24	0.1 μM
RT enzyme	25×	0.48	1×
Subtotal		9.0	
RNA		3.0	
Total		12.0	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free.

2.2.2 AgPath-ID one-step RT-PCR reagents (Applied Biosystems; refs: 4387424-500 reactions or 4387391-1000 reactions).

2.2.3 RT-PCR cycling parameters: reverse transcription at 45°C for 10 min; denaturation at 95°C for 10 min; 45 cycles consisting of denaturation at 95°C for 15 s, annealing and elongation at 60°C for 60 s.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue, host tissue spiked with the target organism, or lyophilized infected plant material).
- Negative amplification control (NAC): molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of

nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, or a synthetic control (e.g. cloned PCR product[5]). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results

Verification of the controls

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should give no amplification

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4 Performance criteria available

No validation data is available.

Appendix 6 – One step conventional RT-PCR (Zeidan, unpublished)

1. General information

1.1 This test is used for specific detection and identification of TRSV, using primers designed by M. Zeidan (PPIS, Israel). The test has been successfully applied on flower bulbs, leaf material of ornamentals and vegetables and green scrapings of twigs of fruit trees. The protocol has been in use since 2005.

1.2 This test has been successfully used for testing bulbs and leaves of *Anemone* sp., *Gladiolus* sp., *Hyacinthus* sp., *Iris* sp., *Lilium* sp., *Narcissus* spp., *Tulipa* sp., leaf material of Cucurbitaceae, *Dianthus* sp., *Glycine* sp., *Mentha longifolia*, *Nicotiana* sp., *Pelargonium* sp., and green scrapings of twigs of *Malus* sp., *Prunus cerasus*, *Vaccinium* sp. and *Vitis vinifera*.

1.3 The primers are located in the CP gene on RNA 2, and produce an amplicon of approximately 650 bp, covering nucleotides 3036–3683 in TRSV RNA 2 complete sequence, NCBI reference sequence NC_005096.1.

1.4 Oligonucleotides

Primer	Sequence
TRSV-F-Zeidan	5′-GCCTCTAAGGACGCAACTGTGACGCTC-3′
TRSV-R-Zeidan	5′-CACAAGCAGCTGACA GACAGACATTC-3′
Nad5-F-Menzel*	5′-GATGCTTCTTGGGGCTTCTTGTT-3′
Nad5-R-Menzel*	5′-CTCCAGTCACCAACATTGGCATAA-3′

*From Menzel *et al.*, 2002.

1.5 The test has been successfully performed on a PTC-200 DNA Engine thermal cycler (BioRad).

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 One gram of plant material is homogenised in 10 mL homogenization buffer (Bioreba general extraction solution) plus 0.2 g sodium meta-bisulfite (Sigma 255556) in disposable extraction bags (Agdia, Bioreba).

2.1.2 Two millilitres of the homogenate is transferred into a microfuge tube and centrifuged for 1 min at 13 000 rpm.

2.1.3 RNA is extracted from 0.2 mL of the homogenate after centrifugation by using the AccuPrep® Viral RNA Extraction Kit (Bioneer), RNeasy Mini Kit (Qiagen), or other kit. After addition of 0.4 mL of VB binding buffer or RLT buffer, respectively, the mixture is incubated for 10 min at room temperature, and further steps are carried out following the manufacturer's instructions.

2.1.4 Total RNA is eluted in 50 µL of storage buffer. It should be used immediately or stored refrigerated for short-term storage (<8 h) or at –80°C for longer periods.

2.1.5 Homogenized plant material (2 mL) can be stored at –80°C for RNA extraction at a later date. Extracted RNA should be stored refrigerated for short-term storage (<8 h) at –20°C (<1 month), or at –80°C for longer periods.

2.2 One-step reverse transcription PCR

2.2.1 Master mix (simplex reaction)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	15.0	N.A.
RT-PCR premix kit (pellet)	10×	N.A.	1×
PCR forward primer: TRSV-F-Zeidan	20 µM	0.5	0.5 µM
PCR reverse primer: TRSV-R-Zeidan	20 µM	0.5	0.5 µM

(continued)

[5]Laboratories should take additional care to prevent risks of cross-contamination when using cloned PCR products.

Table (*continued*)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Subtotal		16.0	
Genomic RNA extract		4.0	
Total		20.0	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 Master mix (duplex reaction)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	14.0	N.A.
RT-PCR premix kit (pellet)	10×	N.A.	1×
PCR forward primer : TRSV-F-Zeidan	20 µM	0.5	0.5 µM
PCR reverse primer : TRSV-R-Zeidan	20 µM	0.5	0.5 µM
PCR forward primer: nad5-F-Menzel	20 µM	0.5	0.5 µM
PCR forward primer: nad5-R-Menzel	20 µM	0.5	0.5 µM
Subtotal		16.0	
Genomic RNA extract		4.0	
Total		20.0	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free.

2.2.2 One-step RT-PCR using Maxime RT-PCR premix kit (INtRON Biotechnology) containing a pellet of 10× RT-PCR buffer, dNTPs, OptiScript RT-system and Hotstart i-star Taq DNA polymerase.

2.2.3 RT-PCR cycling parameters: reverse transcription at 45°C for 45 min; denaturation at 95°C for 5 min; 40 cycles consisting of denaturation at 95°C for 30 s, annealing at 56°C for 1 min, elongation at 72°C for 1 min; terminal elongation at 72°C for 5 min.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC): Bioreba homogenization buffer.
- Positive isolation control (PIC): dried tissue of TRSV-infected plants, commercially available from DSMZ.
- Negative amplification control (NAC): molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC): for example, *in-vitro* transcribed RNA from a cloned TRSV fragment.1
- Internal control (IC): Nad5 can be used as internal control, in a separate reaction as well as in duplex format.

3.2 Interpretation of results

Verification of the controls

- NIC and NAC should produce no amplicons
- PIC, PAC should produce amplicons of approximately 650 bp
- IC should produce amplicon of approximately 180 bp (if applicable)

When these conditions are met:

- A test will be considered positive if amplicons of approximately 650 bp are produced
- A test will be considered negative if it does not produce a band of 650 bp. Tests should be repeated if any contradictory or unclear results are obtained

4 Performance criteria available

No specific validation data is available.